*Research Journal of Recent Sciences* _____ ISSN 2277-2502
Vol. **2(2),** 1-9, February **(2013)** *Res.J.Recent Sci.*

# Genetic and Phytochemical analysis of Cluster bean (*Cyamopsis tetragonaloba* (L.) Taub) by RAPD and HPLC

**Sharma Anubhuti[1]* and Sharma Pratibha[2]**
[1]Biochemistry (ICAR), Vivekanand Parvatiya Krishi Anusandhan Sansthan, Almora, Uttarakhand, INDIA
[2]Department of Bioscience & Biotechnology, Banasthali University, Banasthali-304022, Rajasthan, INDIA

**Available online at: www.isca.in**
Received 7th June 2012, revised 19th August 2012, accepted 8th December 2012

## Abstract

*In recent years chemoprofiling and molecular phylogenetic studies have received considerable attention and used simultaneously in higher plants to characterise the plant species. Guar or cluster bean, (Cyamopsis tetragonoloba (L.) Taub) is a drought-tolerant annual legume crop. India is the world-leader for cluster bean production as it contributes 80% shares of its total production. The aim of the present work is to determine and evaluate the degree of polymorphism in cultivars grown in Rajasthan using RAPDs and to explore the correlation among RAPD and chemical markers in five varieties of guar RGC-936, 1002, 1003, 1031 and 1017 were taken. Phenolic acids such as sinapic acid, cholorogenic acid, caffeic acid and gallic acids were detected among all cultivars. Whereas flavanoids i.e. kaempferol and myricetin were showing variations among all cultivars. Polyphenols are powerful antioxidant and flavonoids play role in the prevention of degenerative diseases such as cancer and cardiovascular diseases is emerging. The phytochemical analysis of guar may expand its nutraceutical and pharmaceutical utilization and information from this study will be useful to breeding programmes for improving guar seed quality.*

**Key words**: Chemoprofiling, *Cyamopsis tetragonoloba*, Guar, HPLC, phenolic acids.

## Introduction

*Cyamopsis tetragonoloba* (L.) Taub commonly known as guar is a cash crop of the family Leguminosae[1]. Guar seed is highly valued in numerous industries because of its galactomannan rich endosperm. Guar galactomannan is also known as guar gum and is used as a viscosity enhancer for both food and nonfood purposes. The galactomannan is found in the endosperm, which makes up about 35% of the dry weight of the seed, 80-90% being pure galactomannan.

None of the legume species with large galactomannan containing endosperms have been reported to have been genetically transformed. The numerous reports of transgenic plants of various legumes clearly show that transformation of legumes is very difficult, even to a scientist skilled in the art. This is further evidenced by the fact that of the approximately 100 legume species of commercial interest less than 5 species have been transformed. For successful transformation plant species should be well characterized. In current scenario, the DNA markers become the marker of choice for the study of crop genetic diversity has become routine, to revolutionized the plant biotechnology. These markers are useful and are reliable indicators of genome structure and function in evolutionary different plant species. Among various molecular markers RAPD is most common which have been extensively used in genetic diversity analysis[2], characterizing germplasm[3], studying inter-genetic and intra-genetic variations of wild populations[4], gene tagging for molecular breeding[5], for genetic mapping of crop plants[6].

Plants produce several secondary metabolites and phenolic acids are very important among them. Phenolic acids are considered to be powerful antioxidant[7]. Polyphenols have been reported to demonstrated antibacterial effect[8], antimutagenic effect, anti-inflammatory, antiproiferative and vasodilatory actions[7]. Chemical profiling establishes a characteristic chemical pattern for a plant material. The determination of phenolic acids is important both for their characterization and to facilitate more efficient uses of the important plant resources[9,10]. Integration of chemo type driven fingerprinting with genotype driven molecular technique is necessary for optimal characterization of plant species[11].

Thus there is a need to elucidate the biochemical pathway in Guar and identify new flavonoids and establish a correlation between molecular and phytochemical pattern in guar from different accession zones so as to develop transformed plant. Identification of DNA markers that can co-relate DNA fingerprinting data with quantity of selected phtyochemical markers associated with that of particular plant would have extensive applications in quality control of raw materials. In the present study intraspecific diversity was assessed by RAPD, which was considered for the first time as a preliminary genetic method by which we can elucidate the correlation between the genetic characteristic bands, and ecological and chemical factors of *Cyamopsis tetragonoloba.*

## Material and Methods

**Plant Material:** Seeds of five different varieties of guar i.e. RGC-936, 1002, 1003, 1031, 1017 were collected from Krishi Vigyan Kendra, Banasthali. These were grown in green house and healthy leaves were collected for the extraction of DNA and extraction of polyphenols.

**DNA Isolation:** DNA extraction from healthy leaves of all five varieties was carried out by using the CTAB method of Doyle and Doyle[12] with slight modifications. The DNA was extracted by using higher concentration of 2- mercaptoethanol, which was subjected to an additional step of purification with chloroform: iso-amyl alcohol treatment followed by precipitation with chilled solution of ethanol-sodium acetate.

**Random Amplified Polymorphic DNA (RAPD) analysis:** The PCR conditions for RAPD marker analysis reported by Williams et al.[13] was optimized with guar template DNA. RAPD analysis was performed by using 44 amplification cycles, annealing temperature $37^0$C and final extension step on $72^0$C for 5min. with fifteen random primers.

**Data Analysis:** The pair wise genetic similarities among all pairs of samples were estimated with Jaccard's coefficient[14] and similarity matrix was constructed. This matrix was subjected to unweighted pair-group method for arithmetic average analysis (UPGMA) to generate dendrogram using average linking procedure. All these computations were carried out using NTSYS-PC software[15].

**Extraction of secondary metabolites:** The extraction was carried out using method given by Bray and Thorpe[16]. Fresh leaves of selected plants were homogenized in methanol using mortar and pestle. After centrifugation supernatant was collected and pellet was discarded.

**Quantitative estimation of secondary metabolites:** Total amount of polyphenols was estimated by using Folin-ciocalteau's reagent and supernatant of each plant, Tubes were mixed and incubated at room temperature for 1 hour. Absorbance was taken at 725 nm against Folin-ciocalteau's reagent and double distilled water as blank.

**Chemoprofiling:** Concentrated extract of phenolic acid was filtered using millipore filter (0.2µm). The 20µl of sample was injected into injection loop. The samples were analyzed by HPLC (Shimadzu-LA), using solvent A (orthophosphoric acid) and solvent B (orthophosphoric acid, glacial acetic acid, acetonitrile). Chromatography was performed on reverse phase C-18 column. A maximum pressure of 400 kgf/cm$^2$ was maintained with solvent A: solvent B ratio of 30:70 at a wavelength of 320nm. Quantification of individual peaks was achieved by comparison to the sample internal standards. Identification of the chromatographic peaks was performed by comparison to known standards: cinnamic acid, p-coumaric acid, o-coumaric acid, sinapic acid, ferulic acid, gallic acid, chlorogenic acid, kaempferol, myricetin and caffeic acid.

**Table-1**
**HPLC of standard compounds and their retention times**

| S. N. | Standard compounds | RT |
|---|---|---|
| 1. | Caffeic acid (CA) | 4.33 |
| 2. | Chlorogenic acid (CL) | 5.192 |
| 3. | Cinnamic acid (CN) | 5.725 |
| 4. | Ferulic acid (FA) | 3.517 |
| 5. | Kaempferol (K) | 7.508 |
| 6. | Myricetin (M) | 9.183 |
| 7. | o-Coumaric acid (o-CA) | 3.592 |
| 8. | p-Coumaric acid (p-CA) | 3.583 |
| 9. | Sinapic acid (SA) | 3.442 |
| 10. | Gallic acid (GA) | 3.692 |

**Table-2**
**Yield of polyphenols in different cultivars**

| S. No. | Cultivars | O.D. at 725nm | Total polyphenol content (O.D. units/gm leaves) |
|---|---|---|---|
| 1 | RGC-1017 | 0.066 | 3.53 |
| 2 | RGC-1003 | 0.069 | 3.9 |
| 3 | RGC-1002 | 0.092 | 7.2 |
| 4 | RGC-936 | 0.076 | 5.18 |
| 5 | RGC-1031 | 0.172 | 9.02 |

**Table-3**
**Major compounds in different cultivars of cluster bean in µg.gfw$^{-1}$ of leaf tissue**

| Sr. no. | RT | Probable identification | RGC 1017 | RGC 1003 | RGC 1002 | RGC 936 | RGC 1031 |
|---|---|---|---|---|---|---|---|
| 1 | 2.6 | Nil | 17.1 | - | 23.2 | - | 18.1 |
| 2 | 3.1 | Nil | 7.75 | 7.51 | 7.8 | 7.93 | 8.1 |
| 3 | 3.4 | Sinapic acid | 6.01 | 5.72 | 6.9 | 5.6 | 6.05 |
| 4 | 3.5 | Ferulic acid | - | - | - | 16.5 | - |
| 5 | 3.7 | Gallic acid | 22.7 | 16.2 | 8.75 | 16.7 | 16.7 |
| 6 | 4.2 | Caffeic acid | 16.9 | 14.9 | 17.1 | 17 | 17.7 |
| 7 | 4.5 | Nil | 12.5 | 14.5 | 14.7 | 14.6 | 12.9 |
| 8 | 5.1 | Cholorogenic acid | 22.3 | 17 | 16.8 | 17.6 | 19.7 |
| 9 | 7.1 | Kaempferol | 28.6 | 27.7 | 29.3 | 28.9 | 41.6 |
| 10 | 9.1 | Myricetin | 20.6 | 14.3 | 12.3 | 26.4 | - |

**Table-4**
**HPLC determination of phenolic compounds (%) in cluster bean cultivars**

| Sr. no. | Standard | RGC 1017 | RGC 1003 | RGC 1002 | RGC 936 | RGC 1031 |
|---|---|---|---|---|---|---|
| 1. | Sinapic acid | 4.4 | 4.8 | 5 | 5.7 | 6.09 |
| 2 | Ferulic acid | - | - | - | 17 | - |
| 3 | Gallic acid | 16.9 | 13.7 | 6.3 | 17.2 | 16.8 |
| 4 | Caffeic acid | 12.6 | 12.6 | 12.4 | 17.5 | 17.8 |
| 5 | Cholorogenic acid | 16.6 | 14.4 | 12.2 | 18.1 | 19.8 |
| 6 | Kaempferol | 25.4 | 23.5 | 21.4 | 29.7 | 38.4 |
| 7 | Myricetin | 21.3 | 12.1 | 8.9 | 27.2 | - |

## Results and Discussion

DNA from leaves of five varieties of cluster bean was extracted using the protocol of Doyle and Doyle with slight modifications; single band purity was obtained by using this method (fig. 2). Ratio of $\lambda_{260}$ and $\lambda_{280}$ was found 1.5-1.7 which indicates the purity of DNA[17,18] (table 6). The yield of DNA was ranges from 15 to 21.6-µg/gm tissue.

A total no. of 15 random decamer primers were used for RAPD analysis (table 5). There were 13 primers with 60% GC content and 2 primers with 70% GC content. Each primer generated a varied no. of fragments, which ranged from 4-12 (figure 3). The maximum average no. of bands (9 bands) generated with primer-10 where as primer-11 generated the minimum average no. of bands (4 bands). RGC-936 and RGC-1017 doesn't showing amplification with primer 11. Whereas there is no amplification in RGC-1002 with primer-7. The percent of polymorphism was calculated which was obtained in the range of 18% to 100%. Maximum Polymorphism is found in primer 11, 13 and 15 (100%) and minimum polymorphism was obtained with primer-14 (table 7). From Jaccard's coefficient, similarity between the cultivars varied from 0.43 to 0.76 with mean value 0.60 evaluated the genetic diversity. Maximum similarity is shown between RGC-1031 and RGC-1017 i.e. 0.76 (76%) where as RGC-936 is 0.43 (43%) similar with RGC-1002, which is showing minimum similarity between cultivars (table 8). Using genetic distance by UPGMA method of clustering, a dendrogram (figure 4) of relationships among the five genotypes was obtained, which is divided into two clusters. Cluster 1 has only RGC-936 cultivar. Cluster 2 contained the remaining four cultivars separated into 3 sub clusters. RGC-1031 and RGC-1017 are in a branch; RGC-1002 and RGC-1003 are in a separate branch. Thus data indicate that RGC-1031 and RGC-1017 are more closely related than others.

The quantitative analysis of total phenolics, extracted from the leaves of cluster bean was done. Table 1 represents the amount of total phenolics in terms of OD units gfw$^{-1}$. Total phenolic content in all cultivar was in the range of 3.5 OD units.gfw$^{-1}$ to 9.02 OD units.gfw$^{-1}$. The highest level of total phenolics was recorded in RGC-1031 cultivar i.e. 9.02 OD units.gfw$^{-1}$ on the other hand, the phenolics level were particularly low in RGC-1017, where the amount of total phenolics was 3.53 OD units.gfw$^{-1}$.

Further methanolic extract of leaves of all cultivars was used for separation of different phenolic compounds by HPLC. The peaks were identified by comparing the retention time (RT) of the standard phenolic acid with the samples (table 2). Number of peaks in chromatograms obtained gives the number of phenolic acids present in the sample. The peak number and corresponding area determines the number of compounds present in the injected samples and their quantity respectively. The distributions of phenolic acids in different cultivars of cluster bean are shown in figure-1. Chromatograms indicate the

variation of different phenolic acids in all these cultivars. It has been found that sinapic acid, cholorogenic acid, caffeic acid, gallic acid were the most widely represented among all phenolic acids in cluster bean. The concentrations of each compound (identified/ unidentified) were calculated on the basis of peak area. The amount of different phenolic compounds in $\mu g.gfw^{-1}$ are summarized in table 3. The amount of caffeic acid in *Zizyphus mouritiana* was reported as 19 $\mu g.gdw^{-1}$ [19]. Conversely, the lower amount of caffeic acid reported[20] in pomegranates was 0.78 $mg.l^{-1}$. Similarly, the amount of caffeic acid in all cultivars of guar varied from 14.9 $\mu g.gfw^{-1}$ in RGC-1003 to 17.7 $\mu g.gfw^{-1}$ in RGC-1031. Whereas ferulic acid was observed only in RGC-936 (16.5 $\mu g.gfw^{-1}$). Gallic acid also varied in all cultivars from minimum 8.75 $\mu g.gfw^{-1}$ in RGC-1002 to maximum 22.7 $\mu g.gfw^{-1}$ in RGC-1017**.** In contrast to other polyphenols little variation was observed in case of sinapic acid i.e. from 5.72 $\mu g.gfw^{-1}$ in RGC-1003 to 6.9 $\mu g.gfw^{-1}$ in RGC-1002**.**

Flavonoids are the naturally occurring polyphenols representing one of the most prevalent classes of compounds in medicinal herbs such as *Silybum marianum*, *Alpina officinarum*, *Hypericum perforatum* and also in vegetables, nuts, fruits and beverages such as coffee, tea and red wine[21,]

Epidemiological studies have shown the protective role of flavonoids against various cancers and more particularly hormone related cancers[22]. In this study few cultivars were showing higher concentration of flavonoids for example Kaempferol was present in relatively higher concentration than other phenolics. It was lowest 27.7 $\mu g.gfw^{-1}$ in RGC-1003 and highest 41.6 $\mu g.gfw^{-1}$ in RGC-1031. These values are in agreement with the reports of various studies. Higher concentration of kaempferol reported in guar leaves[23] as well as in guar seeds[24]. Thus it can be concluded that kaempherol was are the major constituent of these guar cultivars then other phenolics such as sinapic acid, caffeic acid , gallic acid, ferulic acid, chlorogenic acid and myricetin (table 4).

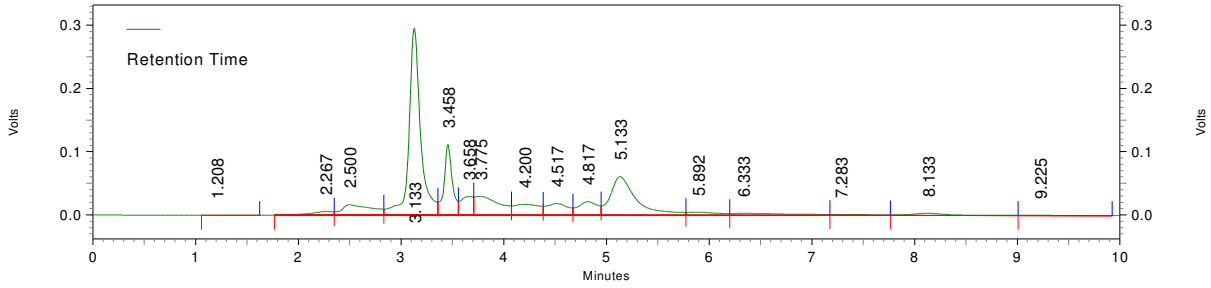
Peak with retention time of 3.10 was most common among all cultivars but it could not be identified, as its retention time did not match with available standards (table 2). Concentration of this unidentified compound among all cultivars was 7.5 $\mu g.gfw^{-1}$ to 8.1 $\mu g.gfw^{-1}$.

**Table-5**
**Arbitrary 10 mer primers used for RAPD**

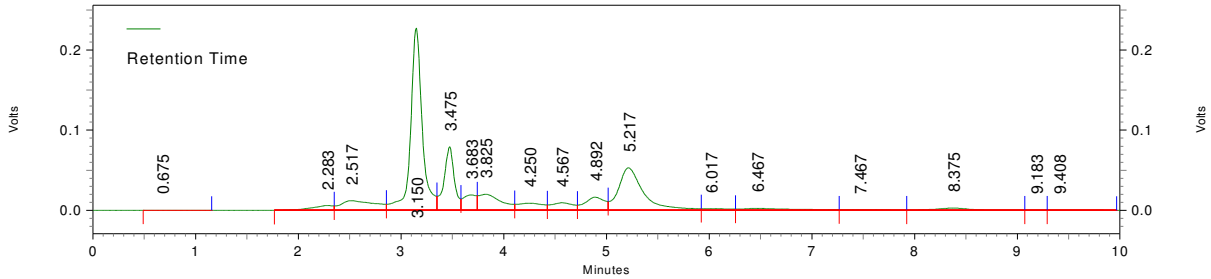
| S.NO. | Sequence of the primer | %GC of Primer | Tm |
|---|---|---|---|
| 01 | 5'- AAAGCTGCGG -3' | 60 | 32 |
| 02 | 5'- AACGCGTCGG -3' | 70 | 34 |
| 03 | 5'- AAGCGACCTG -3' | 60 | 32 |
| 04 | 5'- AATCGCGCTG -3' | 60 | 32 |
| 05 | 5'- AATCGGGCTG -3' | 60 | 32 |
| 06 | 5'- ACACACGCTG -3' | 60 | 32 |
| 07 | 5'- ACATCGCCCA -3' | 60 | 32 |
| 08 | 5'- ACGGAAGTGG -3' | 60 | 32 |
| 09 | 5'- ACCGCCTATG -3' | 60 | 32 |
| 10 | 5'- ACGATGAGCG -3' | 60 | 32 |
| 11 | 5'- ACGGCAACCT -3' | 60 | 32 |
| 12 | 5'- ACGGCAAGGA-3' | 60 | 32 |
| 13 | 5'- ACTTCGCCAC -3' | 60 | 32 |
| 14 | 5'- AGGCGGGAAC-3' | 70 | 34 |
| 15 | 5'- AGGCTGTGTC -3' | 60 | 32 |

**Table-6**
**DNA obtained from different varieties of guar**

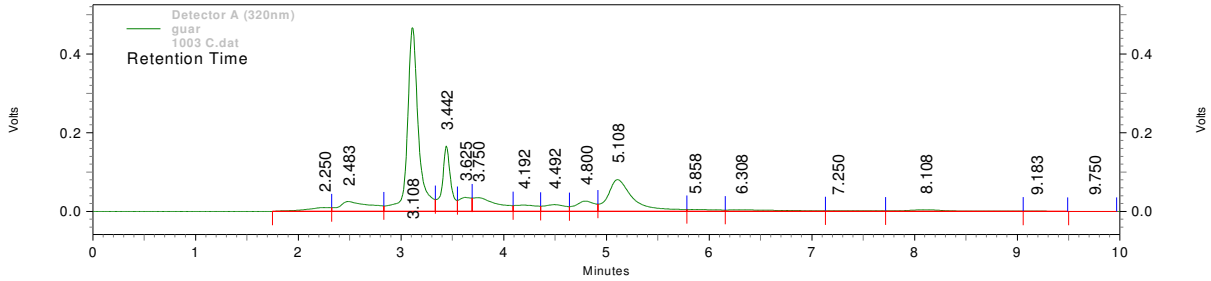
| Varieties | OD260 | OD280 | $\lambda_{260}/\lambda_{280}$ | Conc. (µg/ µl) | DNA yield µg/gm tissue |
|---|---|---|---|---|---|
| RGC-936 | 0.045 | 0.027 | 1.6 | 0.225 | 15.0 |
| RGC-1002 | 0.065 | 0.042 | 1.54 | 0.325 | 21.6 |
| RGC-1003 | 0.055 | 0.034 | 1.6 | 0.275 | 18.3 |
| RGC-1031 | 0.060 | 0.040 | 1.5 | 0.300 | 20.0 |
| RGC-1017 | 0.055 | 0.032 | 1.7 | 0.275 | 18.3 |



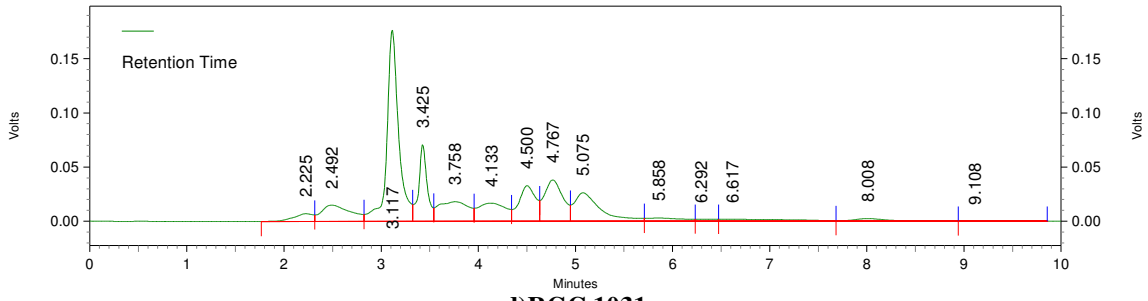

**a) RGC-936**



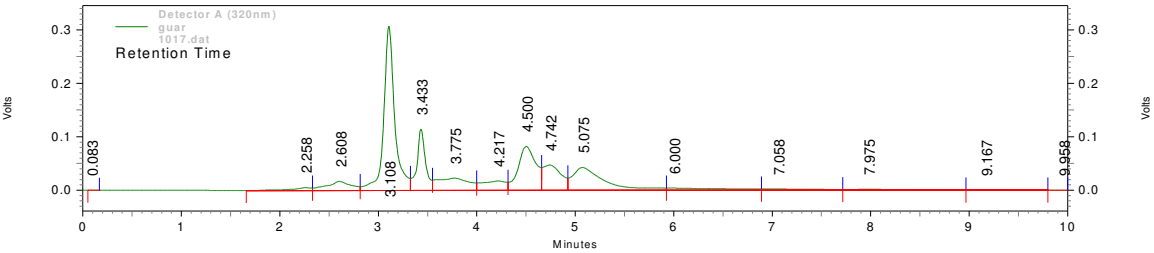

**b) RGC 1002**




**c) RGC 1003**




**d)RGC 1031**




**e)RGC 1017**

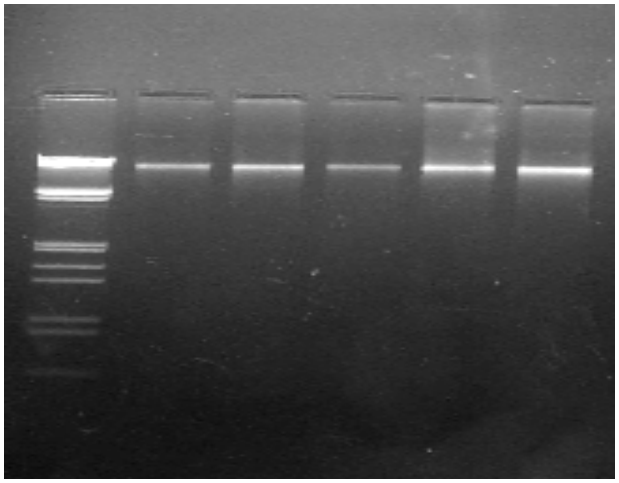
**Figure-1 (a,b,c,d,e)**
**Chromatogram of different cultivars of Guar**

**Table-7**
**Total number of amplified fragments and number of polymorphic fragments generated by PCR using selected random decamers in five varieties of guar**

| No. of Primers | Sequence of Primer | % GC | Tm | Amplication products | Total No. of polymorphic products | No. of Polymorphism |
|---|---|---|---|---|---|---|
| 01 | 5'- AAAGCTGCGG -3' | 60 | 32 | 11 | 10 | 90 |
| 02 | 5'- AACGCGTCGG -3' | 70 | 34 | 9 | 5 | 55 |
| 03 | 5'- AAGCGACCTG -3' | 60 | 32 | 7 | 4 | 57 |
| 04 | 5'- AATCGCGCTG -3' | 60 | 32 | 10 | 8 | 80 |
| 05 | 5'- AATCGGGCTG -3' | 60 | 32 | 7 | 2 | 28.5 |
| 06 | 5'- ACACACGCTG -3' | 60 | 32 | 9 | 5 | 55.5 |
| 07 | 5'- ACATCGCCCA -3' | 60 | 32 | 9 | 4 | 44.4 |
| 08 | 5'- ACGGAAGTGG -3' | 60 | 32 | 7 | 4 | 57.1 |
| 09 | 5'- ACCGCCTATG -3' | 60 | 32 | 8 | 3 | 37.5 |
| 10 | 5'- ACGATGAGCG -3' | 60 | 32 | 12 | 10 | 83.3 |
| 11 | 5'- ACGGCAACCT -3' | 60 | 32 | 9 | 9 | 100 |
| 12 | 5'- ACGGCAAGGA -3' | 60 | 32 | 9 | 5 | 55.5 |
| 13 | 5'- ACTTCGCCAC -3' | 60 | 32 | 13 | 13 | 100 |
| 14 | 5'- AGGCGGGAAC -3' | 70 | 34 | 11 | 2 | 18.1 |
| 15 | 5'- AGGCTGTGTC -3' | 60 | 32 | 11 | 11 | 100 |
| Average | | | | 9.4 | 6.3 | 66 |

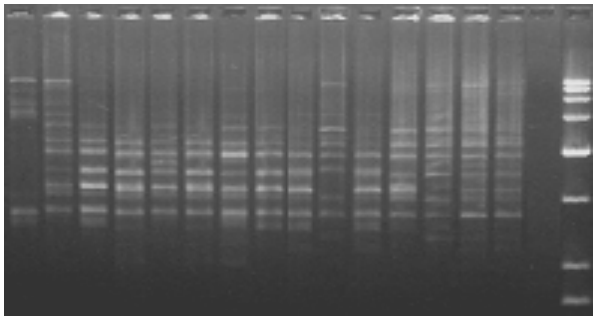
**Table-8**
**Similarity matrix of 5 varieties of *C. Tetragonaloba* based on RAPD profile**

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| RGC- 936 | 1.00 | | | | |
| RGC-1002 | 0.43 | 1.00 | | | |
| RGC-1003 | 0.57 | 0.65 | 1.00 | | |
| RGC-1031 | 0.53 | 0.59 | 0.73 | 1.00 | |
| RGC-1017 | 0.56 | 0.54 | 0.68 | 0.76 | 1.00 |



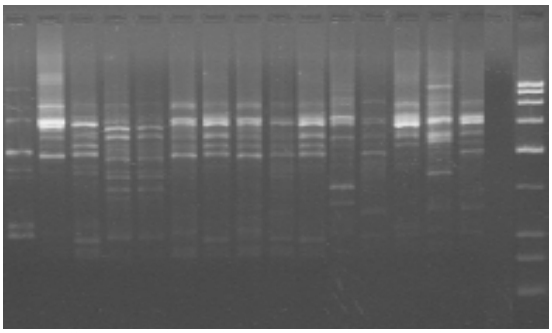
M 1 2 3 4 5

**Figure-2**
**Genomic DNA of RGC-936, 1002, 1003, 1031, 1017 (Lane 1-5), Lambda DNA *EcoR1/HindIII* double digested (Lane M)**



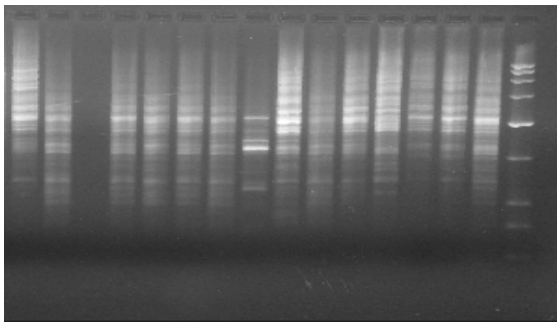
**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

(A)



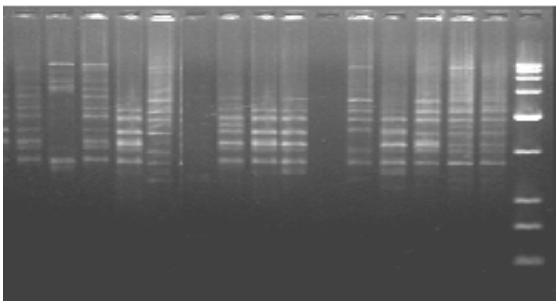
**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

(B)



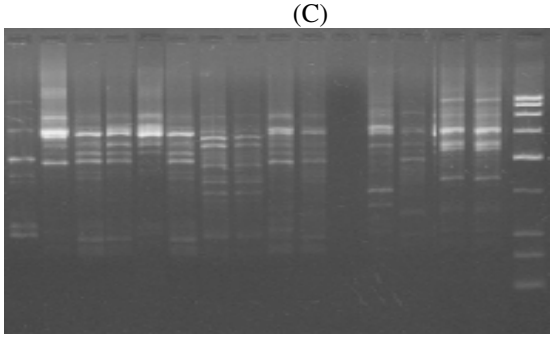
**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

(C)



**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

(D)



**1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16**

(E)

**Figure-3**
**RAPD profiling of five varieties of guar (RGC-936, 1002, 1003, 1031, 1017) with random primers (Lane1-15), DNA 1kb ladder (Lane-16)**

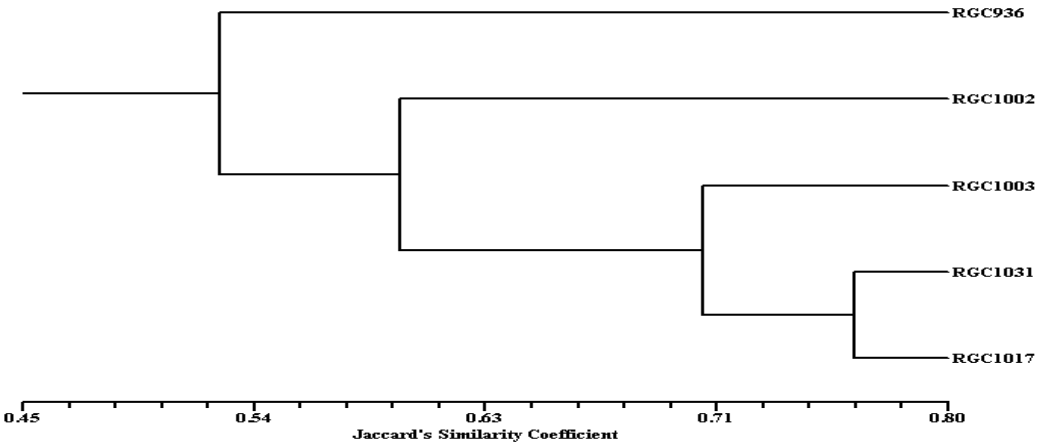



**Figure-4**
**Dendrogram showing diversity of based on RAPD profiling**

## Conclusion

The combination of HPLC and RAPD analysis of plants has been considered by a number of authors. Tanaka *et al.*[25] used RAPD to differentiate the species of *Panax* from each other. They developed a combination of RAPD and eastern blotting analyses using anti-ginsenoside monoclonal antibodies to identify *Panax* spp. Pascal *et al.*[26] studied the significance of polyphenols in Industry. Vieira *et al.*[27] studied morphological, chemical and genetic differences of 12 basil (*Ocimum gratissimum* L.) specimens to determine whether volatile oils and flavonoids could be used as taxonomical markers and to examine the correlation between RAPD and these chemical markers. RAPD analysis has been developed to be a good candidate for the identification of plant species. Gillan *et al.*[28] performed a preliminary study concerning the comparison of *Cannabis sativa* by RAPD and HPLC analysis of cannabinoids. This study could be beneficial to the molecular biologist who are involved in the study of enhancement of Guar gum[29].

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